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(54) Manufacturing process of phospholipideactive principle complexes useful for the liposome preparation.

(57) Process for the preparation of a phospholipid /active principle complex useful for the manufacture of one or more active substances containing liposome, wherein the active principle, intrinsically bound to the phospholipid molecules, is entrapped within liposomes as soon as the phospholipid double layers constitute vesicles by simple aqueous solution addition, comprising the following steps:

- dissolving the active principle(s) into a solvent,
- phospholipid addition to the above solution,
- solvent removing, only in case the solvent should not be present in the final product,
- hydrophilic medium addition to the complex obtained in the previous step to enable the above complex molecules to reorganize in a multilamellar structure having a plurality of bimolecular layers,
- final treatment of the complex to obtain the desired form.

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Background of the invention.

The present invention concerns a process for the manufacture of phospholipid - active principle complexes useful in the subsequent preparations of liposomes exhibiting a high active principle encapsulating efficiency.

The liposome formation mechanism from said complexes, which may be in gel, liquid or powder form, is spontaneous and it takes place simply by complex hydration followed by mechanical stirring.

Liposomes exhibit a structure similar to the one of the cell membranes. It is well known that liposomes are bi-layer phospholipid spherical aggregates that are formed in aqueous solutions as a consequence of a mechanical reorganization of the bimolecular layers in which phospholipids spontaneously organize when dispersed in an aqueous solution. The liposome vesicles may consist of a single membrane phospholipid double layer (SUV- Small Unilamellar Vesicles) or two or more membrane phospholipid double layers (MLV- Multilayer Lipid Vesicles) having their polar heads oriented toward the exterior and toward the vesicle inner aqueous layers.

Liposomes have been used in the past in the pharmaceutical field, nowadays they find an extensive use in the cosmetic industry. The interest of said industry in the use of liposome is due to the fact that liposomes may constitute highly effective systems for the delivery of a broad spectrum of hydro- and lipo-soluble active principles. The hydrosoluble materials may be contained into the liposome inner aqueous spaces and electrostatically bound to the lipid double layer surface. On the other hand the liposoluble materials are retained within the lipid double layer or are sometime entrapped, as minute droplets, within the liposome aqueous zones. The use of liposomes as pharmaceutical and cosmetic active principle carriers has some advantages i.e. a higher treatment efficiency, a reduction of the active principle to be administered, an active principle protection against its deterioration as well as a prolonged release time thereof.

At present many liposome manufacturing methods are known in the art. However most of said methods show, among other drawbacks, the difficulty, and more likely the impossibility, to load the active principles into the liposomes. Therefore the researches in this field aimed to find an effective method to manufacture active principles containing liposomes exhibiting a high encapsulating efficiency.

In order to better illustrate the novelty of the present invention, four kind of techniques are reported hereinafter which are presently employed to manufacture loaded liposomes, i.e. one or more active principle containing liposomes.

The first method, known as passive entrapping, implies the liposome vesicles formation as a consequence of the mechanical stirring of an active principle containing phospholipid aqueous dispersion. The active principle is entrapped into the liposome simultaneously with the liposome formation. Said method has the drawback of a reduced encapsulating efficiency (a large amount of the active principle remaining in the aqueous medium and is not entrapped into the liposome) as well as a variable encapsulating activity depending on the dispersion medium nature, the active principle and other substance concentration.

The second method implies the "solvent evaporation" from a two phase solvent system. Said technique, besides of being unable to provide high encapsulating efficiency liposomes, has the further drawback of promoting the presence into the liposomic solution of undesired organic solvent residues, as for instance, chlorinated hydrocarbons, making them unsuitable for pharmaceutical or cosmetic purposes.

The third technique, making use of a gel consisting of membrane phospholipid double layers, is based on a "two phase dilution". In the first phase an active principle concentrated solution in an insufficient amount for the liposome formation, is mixed with the initial gel. In the second phase the resulting solution is then further diluted with an excess aqueous solution without any active principle. This procedure, even successful in obtaining an active principle encapsulating level higher than the previous techniques, still has, however some disadvantages. First the encapsulating efficiency is no higher than 80% and moreover said efficiency is not constant, but it varies depending on the nature of the active principle. Therefore, to obtain active principle prefixed content liposomes - which is a critical requirement for the use in the pharmaceutical field - a long and expensive set of experimental tests is necessary.

A further disadvantage resides in that the dilution should respect precise values to avoid any active principle release from the liposome inner part to the surrounding dispersing aqueous medium.

The recently disclosed fourth technique, is the so-called "transmembrane loading technique". Said technique, disclosed in PCT/EP 91/02377, involves the preliminary preparation of "empty liposomes", i.e. containing the dispersion aqueous medium only. Thereafter the active principle is introduced into the dispersion medium and, by incubation under controlled temperature and osmolality values, the penetration of the active principle into the core of the liposome will occur. Even if with the above technique it is possible to obtain satisfactory values

of the encapsulating degree, said degree is still variable in function of the nature of the active principle to be encapsulated into the liposomes.

Summary of the invention.

It is an object of the present invention to provide the preliminary preparation of phospholipid and active principle complexes and then "loaded liposomes" - i.e. active principle containing liposome - by a simple and quick method which does not involve the limitations of the prior art techniques.

The invention, as hereinafter described, consists of a process for the manufacture of complexes and active principle containing liposomes characterised by a high encapsulating capacity, close to 100% and the possibility to produce predetermined content liposome both in terms of phospholipids and active principles.

The rationale upon which this system is based consists of the preliminary formation of a complex between the active principle and the phospholipids and then the subsequent arrangement in more double layers of the obtained complex. The invention takes advantage of the phospholipid molecule amphipathy, i.e. the fact that said molecules exhibit a hydrophilic portion and a hydrophobic portion, to create complexes both with hydrophilic and hydrophobic active principles, the first bound to the polar head the second to the hydrophobic tail of the phospholipid.

The phospholipid /active principle complex may be in form of a gel, a liquid or a powder and, by a simple water addition followed by a short mechanical stirring, it allows to obtain liposomes whose active principle, being intrinsically bound to phospholipid molecules, is not merely contained into the liposomic cavity or dissolved in the aqueous phase, but is directly linked to the liposome membrane with an encapsulation degree close to 100%.

Description of the preferred embodiments.

The present invention provides several advantages over the prior art techniques which are illustrated hereinafter.

1. Possibility of preparing phospholipid /active principle complexes having a component stable relationship.
2. High Liposome active principle encapsulating capacity, close to 100% irrespective of the active principle nature. Therefore it is possible to prepare liposome dispersion having a predetermined content both as active principle and phospholipids. Then, the amount of active principle not encapsulated into the liposome and consequently not useful, being unable to cross the epidermal barrier, is, in practice, reduced to zero.
3. Preservative absence in the phospholipid /active principle complex that avoids any risk of preservative substance liposimizing or encapsulating. In case a preservative containing liposomic solution is required, preservatives shall be added afier liposome formation so that they are present in the aqueous dispersion only, but not within liposomes.
4. The possibility of manufacturing apyrogenic, pharmaceutical grade, sterile liposome solution without any preservative addition.
5. Liposome stability, in that no migration of the active principle will occur due to osmotic equilibria involving mechanisms.
6. Possibility of on-the-spot liposome preparation, when required for the use, avoiding therefore any problem connected with liposome conservation and stability. This characteristic enables the preparation of compositions consisting of two components to be mixed when used, that means very stable compositions even in the absence of preservatives useful for the active principle injection or administration to cell cultures.

Convenient active principles useful in the process according to the present invention may vary depending on the sought applications. In the cosmetic field applications the following can be used: anti-wrinkle active principles such as polyunsaturated fatty acids, Echinacea extract, Aloe Vera extract, Fetuin, Vitamin A and the like; free radical scavenging active principles such as Superoxide Dismutase, Peroxidase, Leucocianidins, Vitamin E; cellulitis preventing active principles such as Escin, Ruscogenin, Caffein, Horse-chestnut extract, Carnitin and the like, revitalizing active principles as Cytochrome C, placenta extract, Ginseng extract and the like. In the pharmaceutical field applications the following can be used: antimycotic active principles as thiazole derivatives, Griseofulvin and the like; antibiotic active principles as Penicillin, Vancomycin, Cephalosporin and the like; hormone based active principles as Calcitonin. In the dietetic field applications the following can be used: vitamin and other adjuvant based active principles. In the agronomy field applications the following can be used: herbicide active principles as dinitro-ortho-cresol, carbamates and the like; fungicide active principles as organophosphates, organochlorides, thiophosphates and the like.

Convenient phospholipids for the use in the complex manufacturing process according to the invention can be either of animal and vegetal origin or synthetic. Among the vegetal origin phospholipids soybean lecithin can be employed,

among the animal origin ones egg lecithin and cerebrosides can be used.

A - GEL FORM

PHASE 1 The active principle is dissolved in a suitable solvent;
PHASE 2 Preparation of the complex between the active principle solution resulting from the previous phase and the phospholipids, whose nature and purity level is in accordance with to the ultimate use of the product. If the selected solvent (f.i. hexane) should not be present in the final product it will be removed by vacuum distillation or evaporation.
PHASE 3: A multilamellar phospholipid /active principle structure is obtained by addition of an ethanol based solution until a soft mixture is formed.
PHASE 4: gel form completion by glycerol addition.

B - LIQUID FORM

PHASE 1 AND 2: As indicated under A (gel form)
PHASE 3: A multilamellar phospholipid /active principle structure is obtained by addition of an ethanol based solution in such an amount to yield a liquid product.

C - POWDER

PHASE 1, 2 AND 3: As indicated under A above (gel form).
PHASE 4: The complex is dried under vacuum at a predefined temperature depending on the nature of the active principle.

As aforesaid, to obtain liposomes, it is sufficient to add an aqueous solution to the phospholipid /active principle complex. The volume of the aqueous solution has to be calculated according to the active principle sought concentration in the liposome solution.

The solvent is selected in function of the nature of the active principle(s) to be complexed with phospholipid . When feasible, it is advantageous to use solvents that are not to be removed afterward and may be present in the final product. If it is not possible the undesired solvent is removed from the complex medium, for instance, by evaporation or vacuum distillation.

As an example to illustrate the present invention, the procedures for the preparation of two complexes in gel, liquid or powder form are reported hereinafter. The relevant active principles are soluble in different solvents.

EXAMPLE 1

A - PHOSPHOLIPID /VITAMIN A COMPLEX IN GEL FORM

Vitamin A acetate in the exact calculated amount (10%, 1.000.000 UI/g) was dissolved in hexane (Vit. A / hexane 1:1). The mixture was stirred until a homogeneous mixture is obtained.
PHASE 2: Phospholipid powder (60 %), of the desired nature and purity, was added to the mixture of phase 1, and a homogeneous mixture was obtained. Hexane was removed from the complex under vacuum by increasing the temperature.
PHASE 3: When hexane is completely evaporated, ethanol (10%) is added thereto under stirring to obtain a homogeneous mixture.
PHASE 4: At the end glycerol is added to enhance the gel complex formation.

B - PHOSPHOLIPID /VITAMIN A LIQUID FORM COMPLEX

PHASE 1 and 2: as indicated under A above (gel form)
PHASE 3: When hexane is completely evaporated ethanol is added in such an amount to obtain a liquid product.

C - **PHOSPHOLIPID /VITAMIN A POWDER FORM COMPLEX**

PHASE 1,2 and 3: As indicated under A (Gel form)
PHASE 4: The obtained mixture was vacuum dried at room temperature.

EXAMPLE 2

A - **PHOSPHOLIPID /SUPEROXIDE DISMUTASE (SOD) GEL FORM COMPLEX**

PHASE 1: The desired amount of SOD (1.000.000 UI/Kg) was dissolved in an alcoholic solution (water/ethanol ratio 1:3, amount 20%) under stirring until a homogeneous mixture was obtained.
PHASE 2: Phospholipid powder (70 %), of the desired nature and purity, was added to the mixture of phase 1, and a homogeneous mixture was obtained.
PHASE 3: In the preparation of this complex, as well as any complex whose active principles is water or water-ethanol soluble, phase 3 is not required. Ethanol, necessary for the double-layer liposome formation, is the one used to dissolve the active principle

PHASE 4: At the end glycerol is added to enhance the gel complex formation.

B - **PHOSPHOLIPID /SUPEROXIDE DISMUTASE (SOD) LIQUID FORM COMPLEX**

PHASE 1 and 2: As indicated under A above (gel form)
PHASE 3: Ethanol is added thereto in such an amount to obtain a liquid product.

C - **PHOSPHOLIPID /SUPEROXIDE DISMUTASE (SOD) POWDER FORM COMPLEX**

PHASE 1,2 and 3: As indicated under A (gel form)
PHASE 4: The obtained mixture was vacuum dried at room temperature.

EXAMPLE 3

Measurement of entrapment efficiency.

A gelified phospholipid /Vitamin A complex was prepared according to the procedure described in Example 1A.

Liposomes were then obtained by adding a physiologic solution to the complex and subsequent stirring of the mixture.

The obtained liposomes have been subjected to ultracentrifugation in order to separate the liposome phase from the water phase. In both phases Vitamin A was determined as follows.

Liposome phase:

Phospholipid /vitamin A pellet resulting from the ultracentrifugation was dissolved in hexane, filtered under vacuum ($0.2 \cdot 10^5$ Pa) and determined by HPLC (high performance liquid chromatography) using two different HPLC columns:

1) Column: Chromsep Nucleosil C18 100 mm x 3 ID
Eluent: methanol
Flow: 1 ml/min.
Detecting wl: 325 nm
2) Column: Pertsil 10 ODS
Eluent: Methanol/water (90/10)
Flow: 1ml/min.
Detecting wl: 325 nm

The water phase coming from the ultracentrifugation was analyzed for its vitamin A content using the same HPLC procedure and columns.

No Vitamin A was detected in the aqueous phase while almost all Vitamin A was detected in the liposome phase.

The above test confirms the high entrapment efficiency of the liposome obtained from the complexes manufactured according to the present invention.

Claims

1. Process for the preparation of a phospholipid /active principle complex useful for the manufacture of one or more active substances containing liposome, wherein the active principle, intrinsically bound to the phospholipid molecules, is entrapped within liposomes as soon as the phospholipid double layers constitute vesicles by simple aqueous solution addition, characterized by the following steps:
   - dissolving the active principle(s) into a solvent,
   - phospholipid addition to the above solution,
   - solvent removing, only in case the solvent should not be present in the final product,
   - hydrophilic medium addition to the complex obtained in the previous step to enable the above complex molecules to reorganize in a multilamellar structure having a plurality of bimolecular layers,
   - final treatment of the complex to obtain the desired form.

2. Process according to claim 1, characterized in that the complex multilamellar structure formation takes place by addition of an alcoholic solution.

3. Process according to claim 1 and 2, chatacterized in that the gel form multilamellar structure preparation takes place by ethanol solution addition.

4. Process according to any of the previous claims, characterized in that the complex preparation takes place by mixing said active principle(s) with membrane phospholipids in the presence of a suitable solvent.

5. Process according to one or more of the previous claims, characterized in that the final treatment of the complex consists of a glycerol addition to enhance the gel formation.

6. Process according to claims 2 and 3, characterized in that the complex final treatment consists of an alcohol solution addition until a liquid product is obtained.

7. Process according to claims 1 to 4, characterized in that the complex final treatment consists in drying the same to obtain a complex in

powder form.

8. Process according to any of the previous claims, characterized in that the solvent used for the complex multilamellar structure formation is the same solvent used to dissolve the active principle.

9. Phospholipid complex with one or more active principles prepared by the process according to the present invention, characterized in that the active principles are selected among the ones exhibiting activity in the cosmetic, pharmaceutical, dietetic and agronomic fields.

10. Phospholipid complex with one or more active principles prepared by the process of the present invention, characterized in that the active principles are selected among vitamins, enzymes, antibiotics and hormones and their mixtures.

11. Complex according to claim 9, characterized in that the active principle is Vitamin A.

12. Complex according to claim 9 characterized in that the active principle is Superoxide Dismutase.

13. Complex according to claim 9, characterized in that the phospholipids are of vegetal origin.

14. Compositions for pharmaceutical, cosmetic, dietetic and agronomic use containing the phospholipid /active principle complex according to claims 9 to 13.

15. Liposomes when obtained from the complexes according to the previous claims by aqueous solution addition.

16. Therapeutic and cosmetology compositions for the use in the pharmaceutical, cosmetic, dietetic and agronomic field containing the liposome according to the preceding claim.

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EUROPEAN SEARCH REPORT

Application Number EP 95105736.3

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 6)
X	WO - A - 90/04 961 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) * Claims 1,5,7,8,12; page 10, line 29 - page 11, line 4; page 13, line 30 - page 14, line 20 * --	1,4,7, 9,10, 15,16	A 61 K 9/127 B 01 J 13/00
X	GB - A - 2 013 609 (L'OREAL) * Claims 1,5,13-19; examples 4-6 * --	1,4,7-12,14-16	
X	US - A - 4 830 858 (PAYNE N.I. et al.) * Abstract; claims 1,2,4, 6,8-12,15-18,20,22-28; examples * --	1,4,7, 9,10, 13-16	
X	GB - A - 2 047 535 (A. NATTERMANN & CIE.) * Claims 1,4,5,8,10-12; page 2, lines 13-26; example 2 * --	1,4,7-9,14-16	TECHNICAL FIELDS SEARCHED (Int. Cl. 6) A 61 K 9/00 A 61 K 7/00 B 01 J
X	US - A - 4 311 712 (EVANS J.R. et al.) * Abstract; claims 1-3, 6-10,12; column 2, line 52 - column 3, line 11; example 1 * --	1,4,7, 9,10, 13-16	
X	US - A - 4 963 362 (RAHMAN Y.-E. et al.) * Abstract; claims; examples; column 3, line 60 - column 4, line 2 * --	1,4,7, 9,10, 13-16	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	18-10-1995	MAZZUCCO

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 6)
X	WO - A - 93/23 015 (INSTITUTO NACIONAL DE ENGENHARIA E TECNOLOGIA INDUSTRIAL) * Abstract; claims 1,12,16, 17,24,26,27; page 5, line 10 - page 8, line 1; page 11, line 19 - page 12, line 25 * --	1,4,7-10,14-16	
X	EP - A - 0 317 120 (VESTAR, INC.) * Abstract; claims 1,2,9, 11,13,16,21; page 6, line 50 - page 7, line 46 * --	1,4,7, 9,10, 13-16	
X	US - A - 4 731 210 (WEDER H.G. et al.) * Column 15, lines 13-29; column 9, line 51 - column 10, line 11 * --	1,2,4, 5,7,9, 10,14-16	TECHNICAL FIELDS SEARCHED (Int. Cl. 6)
X	WO - A - 91/16 882 (LIPOSOME TECHNOLOGY) * Claims 1,2,4,7,8; abstract; example 1 * --	1,4,7, 9,10, 12,14-16	
X	US - A - 4 271 196 (SCHMIDT) * Claims; examples 7,9, 13-15; column 3, lines 7-41 * ----	1-4,6, 7,9-11,13-16	

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